State-of-the-art in marketed adjuvants and formulations in Allergen Immunotherapy: a position paper of the European Academy of Allergy and Clinical Immunology (EAACI)

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Abstract: Since the introduction of allergen immunotherapy (AIT) over 100 years ago, focus has been on standardization of allergen extracts, with reliable molecular composition of allergens receiving the highest attention. While adjuvants play a major role in European AIT, they have been less well studied. In this Position Paper we summarize current unmet needs of adjuvants in AIT citing current evidence. Four adjuvants are used in products marketed in Europe: aluminium hydroxide (Al(OH)3) is the most frequently used adjuvant, with microcrystalline tyrosine (MCT), monophosphoryl lipid A (MPLA) and calcium phosphate (CaP) used less frequently. Recent studies on humans, and using mouse models, have characterized in part the mechanisms of action of adjuvants on pre-existing immune responses. AIT differs from prophylactic vaccines that provoke immunity to infectious agents, as in allergy the patient is pre-sensitized to the allergen. The intended mode of action of adjuvants is to simultaneously enhance the immunogenicity of the allergen, while precipitating the allergen at the injection site to reduce the risk of anaphylaxis. Contrasting immune effects are seen with different adjuvants. Aluminium hydroxide initially boosts Th2 responses, while the other adjuvants utilised in AIT redirect the Th2 immune response toward Th1 immunity. After varying lengths of time, each of the adjuvants supports tolerance. Further studies of the mechanisms of action of adjuvants may advise shorter treatment periods than the current three-to-five-year regimens, enhancing patient adherence. Improved lead compounds from the adjuvant pipeline are under development and are explored for their capacity to fill this unmet need.

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All authors have read and approved the position paper. Any potential conflicts of interests are listed here:

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Contributions

JJE coordinated the project, contributed to specific parts below, edited and compiled texts into the final article. KT helped in the ms. coordination and preparation.

part 1 (Introduction) was drafted by JJE and PO; **part 2** (Definition of adjuvant in AIT) was written by KT and KL; **part 3** (The contents of adjuvants) by JJE and RWF; **part 4** (Alum) by RH, KT, JJE, JL, MV and BS; **part 5** (MPL) by PfO, PO, MR, KL; **part 6** (Tyrosine) by KT, RC, SWC; **part 7** (Ca-phosphate and others) by RC, MP, KT; **part 8** (Pipeline) by RWF, PO, KT; part 9 (synopsis) by JJE, KT, BS, MV.

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Key words *(max 5)*
Adjuvants, allergen immunotherapy; aluminium, monophosphoryl-lipid A (MPLA), microcrystalline tyrosine

Abbreviations

Al(OH)3	aluminium hydroxide
APC	antigen presenting cell
CaP	Calcium phosphate
CpG-ODN	cytosine-guanine dinucleotide- oligodesoxynucleotides
CCL-1	CC-Chemokine ligand-1
DCs	dendritic cells
HDM	house dust mite
LPS	lipopolysaccharide
MCT	microcrystalline tyrosine
MPL	monophosphoryl-lipid A
SLIT	sublingual allergen immunotherapy
SCIT	subcutaneous allergen immunotherapy

Abstract

Since the introduction of allergen immunotherapy (AIT) over 100 years ago, focus has been on standardization of allergen extracts, with reliable molecular composition of allergens receiving the highest attention. While adjuvants play a major role in European AIT, they have been less well studied. In this Position Paper we summarize current unmet needs of adjuvants in AIT citing current evidence. Four adjuvants are used in products marketed in Europe: aluminium hydroxide (Al(OH)3) is the most frequently used adjuvant, with microcrystalline tyrosine (MCT), monophosphoryl lipid A (MPLA) and calcium phosphate (CaP) used less frequently. Recent studies on humans, and using mouse models, have characterized in part the mechanisms of action of adjuvants on pre-existing immune responses. AIT differs from prophylactic vaccines that provoke immunity to infectious agents, as in allergy the patient is pre-sensitized to the allergen. The intended mode of action of adjuvants is to simultaneously enhance the immunogenicity of the allergen, while precipitating the allergen at the injection site to reduce the risk of anaphylaxis. Contrasting immune effects are seen with different adjuvants. Aluminium hydroxide initially boosts Th2 responses, while the other adjuvants utilised in AIT redirect the Th2 immune response toward Th1 immunity. After varying lengths of time, each of the adjuvants supports tolerance. Further studies of the mechanisms of action of adjuvants may advise shorter treatment periods than the current three-to-five-year regimens, enhancing patient adherence. Improved lead compounds from the adjuvant pipeline are under development and are explored for their capacity to fill this unmet need.

1. Introduction

Allergen immunotherapy (AIT) is a long-standing and effective intervention to induce tolerance in a hypersensitive patient[1] and it is currently the only disease-modifying, potentially curative treatment option for allergy. Typically, AIT comprises incremental doses to achieve high cumulative doses of allergen extracts, mostly via the subcutaneous (SCIT) and often via the sublingual/mucosal route (SLIT), to induce a state of sustained tolerance[2,3] [4]. Regarding the apparent heterogeneity in the field of AIT and its current coming-of-age transformations, it seemed appropriate and timely to provide a state-of-the-art position paper on adjuvants in AIT. This EAACI-task force thus aimed at providing a current and transparent overview on currently used adjuvants and formulations in AIT, and especially highlights unmet needs.

Mechanistically, AIT counteracts the predominant Th2 immunity in allergy by several well-described immunological mechanisms, altogether resulting in tolerance towards the natural exposure of the allergen. The immunological changes associated with successful AIT include the generation of allergen-specific regulatory T and B cells [5-8], both a source of the immunomodulatory cytokine IL-10[9], and/or CD4 cell subsets including Th1 cells[10], generation of regulatory DCs [11], inhibition of Th2 responses, and reduction of infiltrating inflammatory cells [5]. It may be not necessarily associated with decreases of allergen-specific IgE levels, and the induction of allergen-specific IgA and IgG. The most classical hallmark of AIT is the increase of allergen-specific IgG4, the only anti-inflammatory IgG subclass. Allergen-specific IgG, particularly IgG4 may i) act as a blocking antibody, trapping the allergen before it can crosslink surface-bound IgE on allergy effector cells including mast cells and basophils, for instance as neutralizing antibody in nasal fluids [12]; ii) interact with inhibitory IgG receptor FcγRIIb and downregulate IgE-mediated signalling [13,14]; iii) repolarize macrophages from their allergenic phenotype M2a into tolerogenic M2b, characterized by IL-10 and CCL1 secretion [15]. Disappointingly, none of the described cellular or humoral biomarkers has so far been able to predict the clinical outcome of AIT [16], neither in SCIT nor SLIT [17]. Current publications explicitly aiming to fill this gap recently added several candidates to the list of potential biomarkers, including nasal IgG4 [12], early IL-10 producing B-cells [18], IL-35 [19,20], follicular regulatory T cells [21], or human lipocalin-2, a biomarker for the clinical response in grass pollen and house dust mite SLIT [22].

Overall, it is unequivocal that the AIT products applied in daily practice have clinical efficacy but there are still some drawbacks related to undesired side effects, low efficacy, long treatment duration and patient compliance [4,23,24].

2. Definition of "adjuvant" in AIT

Placebo-controlled studies conducted both in Europe and the United States (US) have supported both the efficacy and safety of a variety of AIT modalities. However, over the decades, the products as well as routine clinical practices of allergists who administer AIT have considerably diverged between the US and Europe [25]. To this end, nearly all products approved for SCIT in the US are aqueous extracts. Compared with aqueous products in the US, adjuvant-absorbed suspensions are preferentially used in Europe which could delay systemic absorption and reduce risk of severe anaphylactic reactions. Furthermore, it has been previously hypothesized that European vaccines "may gain more acceptance because of increasing regulatory approval and lower numbers of injections" [26]. However, head-to-head studies of aqueous versus non-aqueous formulations which could address the relative safety profiles of these products are lacking.

In Europe the allergoid approach has been widely undertaken under the assumption to minimize the risk of side effects. Allergoids are chemically-modified allergens to reduce the IgE binding, but they are usually not applied without adjuvants. In the adjuvants approach, allergens are physically precipitated, creating a depot at the injection site, while simultaneously enhancing immunogenicity (Table 1) (Figure 1). The safety of allergoids [27] allows fast updosing [28] and induction of IL-10 and protective antbodies[29], but this strategy can be corroborated by the choice of adjuvans[30]. In this paper we thus focus on the adjuvants approach, rather than on the allergoid concept. Table 1 also illustrates that the choice of methods presently used for formulation of marketed AIT products is quite limited. Adjuvants also may enhance the efficacy of AIT by polarizing the immune response towards a protective immune response. Adjuvants typically comprise danger signals, leading to inflammation and enhancing the subsequent immune response against the applied allergen. In principle, AIT adjuvants using the TLR-based vacuolar pathway or the aluminium-based cytosolic pathway and lead to enhanced cross-presentation by DCs [31]. Mouse studies indicate that different adjuvants may induce distinct inflammatory signatures: aluminium hydroxide (Al(OH)3) and microcrystalline tyrosine (MCT) via NAPL3 inflammasome activation [32] induce caspase-dependent IL-1beta secretion in a TLR independent manner [33] ; aluminium hydroxide induces a release of IL-5 as an initiator of eosinophilic inflammation; monophosphoryl-Lipid A (MPL) acts via TLR4 induced high levels of TNF-alpha, IL-1 alpha and IL-6 [34]. These divergent immune mechanisms are especially expressed in the onset of AIT and whether these initial effects are per se beneficial or detrimental, or affect the outcomes or efficacy of AIT is not known and should be fully investigated. Therefore, each adjuvant acts via distinct immunological mechanisms, modulating adaptive as well as innate immune responses, all ultimately counteracting the Th2 response, or dampening the allergic inflammation. In marketed

European SCIT products, mostly $Al(OH)_3$, much less frequently MCT, MPL, or other adjuvants are applied, as listed in Table 2 and shown in Figure 2A for grass pollen SCIT as an example.

Besides SCIT (respiratory and venom allergies) and SLIT (respiratory allergies), principally the epicutaneous (in food allergy) [35], intravenous (in drug, biologics and hormone allergy [36]), intralymphatic [37], and oral routes are possible [38]. The adjuvant choice may be decisive for optimally targeting the allergens to the lymphoid organs depending on the route of administration. Adjuvant formulations for subcutaneous, mucosal, and percutaneous AIT applications presently addressed in registered clinical trials and listed in the official databases EudraCT and ClinicalTrials.gov, are presented in Table 3. The overview makes clear that most SCIT trials with allergen extracts, as well as clinical trials with allergoids, hypoallergens, and fusion proteins, use aluminium salts as adjuvants.

In terms of mucosal applications (SLIT, oral and intranasal) and epicutaneous AIT, most preparations in clinical practice do not contain adjuvants. Notably, there is increasing activity in introducing various adjuvant candidates for the mucosal route, like MPL, allergen-conjugates to adjuvants, virus-like particles (VLPs), or particulate allergen delivery systems such as chitin and cellulose, or for percutaneous application polylactic acid or silver particles in clinical trials (Table 3).

However, a plethora of alternative adjuvants and formulations of allergen extracts has been developed and are in the preclinical pipeline awaiting introduction into clinical testing and practice (suppl. Table 1). They may change our way of performing AIT in the future. Immune-modifying platforms such as allergen-displaying VLPs, or cytosine-guanine dinucleotide (CpG)-motifs, adjuvants like MPL combined with Al(OH)3 or with MCT, or spiking of molecular allergens with natural micronutrients, may improve the efficacy of AIT and more efficiently direct the immunological response toward a protective response or immunological tolerance [39]. Delivery systems, such as liposomes and microspheres as well as adjuvants such as Toll-like-receptor agonists [e.g. nonmethylated CpG-motifs derived from bacterial DNA] have been tested in clinical phase II and III trials demonstrating encouraging clinical effects [40] (suppl. Table 1). A high-density display of allergens on virus-like particles enhances the immunogenicity and at the same time seems to reduce potential anaphylactic reactions [41].

The assessment of efficacy and safety of AIT adjuvants and formulations is hampered by the lack of head-to-head comparison studies, or inclusion of placebo controls in trials. Dose finding studies are on-going to fulfil the EMA-Guideline on the Clinical development of products for SIT for the Therapy of Allergic Diseases (CHMP/EWP/18504/2006) [42]. These studies –together with ongoing activities of allergen standardization- will improve insight in efficacy and safety of

allergen products and may pave the way for a transparent declaration of allergen dosages and extract composition, but also of adjuvants.

3. Adjuvants in marketed products

To enable a direct comparison among adjuvants and formulations currently used in AIT trade products on the market, a survey of the industry was performed specifically addressing the compositions for SCIT with grass pollen extracts. Leading pharmaceutical companies with marketed grass pollen products were approached by letters in November 2017, and asked to fill-in the supplied Tables to be published in this position paper of EAACI. Responses filled-in in the Tables (Table 2 and suppl. Table 2) were gathered between December 2017 and March 2018 and were included in unmodified form (as stated by the companies) in the Tables. All industry representatives supplying the data were informed about our parallel request to all competing companies. Eight of 11 approached companies responded and listed single to several products, totalling 21: AllergoPharma (Allergovit®), ALK (Alutard SQ; ALK7/Start SQ; AVANZ®; Pangramin Ultra; Aquagen 100; IRIS), Allergy Therapeutics (Pollinex® Grass, TA Graser top, MATA PFS; Pollinex® Quattro Grass/MPL; Pollinex® Quattro Grass/MCT; Tyrosine TU top Grass), HAL Allergy BV (Purethal® grasses), LETI (Depigoid grass; Depiquick), LOFARMA SpA (LAIS-in), ROXALL (CLUSTOID; Deposit; Allergovac Depot; Allergovac Polimerizado), STALLERGENES (Alustal®; Phostal®), as collectively illustrated in Table 2. ANERGIS SA, ASIT biotech and Biomay AG, at the time of the survey, had no grass pollen product on the market.

The classical adjuvant Al(OH)3 was used in 14 of 21 listed grass pollen SCIT products, Calcium phosphate (CaP) in two, MCT in three, MPL in one (Figure 2A). Further, mannitol was contained in two products, phenol in five, which serve as stabilizers rather than adjuvants.

The number of injections depended on the schedules (3-5 years) and ranged from n=18-63 (mean n=30) in formulations with Al(OH)3 (suppl. Table 2), between n=39-63 (mean 51) in one of the two products adjuvanted with CaP, between n=18-36 (mean n=24) for products with MCT, and n=12 for the single product adjuvanted with MPLA (Figure 2B; suppl. Table 2).

In most cases, the ratio of allergen : adjuvant is not declared or not known (Table 2 and suppl. Table 2). In a single case, the precise ratio of group 5 allergens to adjuvant is revealed in the columns where we requested mg/mg declaration. The nomination of major allergens is advantageous as allergen extracts also contain non-protein compounds, and non-allergens which do not contribute to the specific activity of the extract. Therefore, instead of whole protein, or weight-by-volume (Noon unit), the protein nitrogen unit (PNU; quantity of nitrogen extractable from 1 µg of pollen) was introduced. Today, measures reflecting the biological activity of the extracts, such as histamine equivalent in prick testing (HEP), biologic unit (BU), or bioequivalent

allergen units (BAU) have been suggested for optimization of allergen standardization [43]. It is, however, challenging to determine this activity for allergoids. The need of consistent quality in manufacturing for reliable composition of allergen extracts in terms of major and minor allergens, is increasing and supported by novel standardization methods [44].

4. Aluminium compounds

Aluminium hydroxide is the most widely used adjuvant for SCIT introduced since 1937, and aluminium and its chemical derivatives strongly support the immunogenicity of antigens [45] [46]. Since it is the oldest adjuvant in AIT, most facts, figures and toxicity studies are available for Al(OH)3. Mechanisms involved include both a depot effect (i.e. slow release of the allergen, formulation of the allergen as particles to target antigen presenting cells (APCs)) as well as interaction with the innate immune system: e.g. by stimulating the release of damage-associated molecular patterns (DAMPs) [47], by activating inflammatory DCs[48] or by metabolic reprogramming DCs [30], while the previously reported dependence from inflammasome activation[32] recently was disputed[33]. More than 90% of all registered AIT products contain Al(OH)3 in which the European Pharmacopoiea limits the aluminium content to 1.25 mg per parenteral dose [49]. The products currently registered (in Germany) contain 0.113 to 1.135 mg/ml Al(OH)3, during up-dosing and maintenance phase of SCIT. The therapeutic allergens are adsorbed to the adjuvants and Al(OH)3 is characterized by a high degree of insolubility; this is actually a wanted effect since it results in a depot of the therapeutic allergen and increases the therapeutic success of AIT. The consequences are slow resorption and delayed bioavailability of the antigen. A study in rabbits using radioactive labelled Al(OH)3 demonstrated that within 28 days 17% of the intramuscularly applied Al(OH)3 was resorbed and 6% was excreted through the kidney [50]. A maximal concentration of 2 μg/l of aluminium was observed in plasma. In one study, rabbits were subjected to 20 subcutaneous applications of aluminium lactate [51]. The *no observed effect level* (NOEL) was calculated to be 0.7 mg/kg per day. Interspecies extrapolation yielded a human equivalent dose of 23 mg aluminium for a 70 kg adult, which is more than 20-fold higher than the aluminium dose in a single shot in available therapeutic allergen preparations. Presently, novel *in vitro* methods are developed to test the toxicity of aluminium [52,53], for instance the Paul-Ehrlich-Institute (PEI, Langen, Germany) develops toxicokinetic models in vitro, -in silico, and in rats to determine intramuscular absorption of Al(OH)3 for a risk prediction in humans [54], [55].

Among all pharmaceutical, occupational and consumer exposures potentially representing a health risk, the primary source of aluminium exposure in humans is the food [56]. There is a large

inter-regional variation in the daily aluminium uptake, and a range between 0.2 to 1.5 mg/kg per week was calculated for adults. For children a maximal dose of 0.7 to 2.3 mg/kg per week was reported by the European Food Safety Authority in a news release [57]. The health risk of aluminium originating from food has been evaluated several times by international experts including the Joint FAO-WHO Expert Committee on Food Additives (JECFA) and the AFC Panel (panel on food additives flavourings processing aids and materials in contact with food) of the EFSA [58,59]. EFSA calculated the *tolerable weekly intake* (TWI) of aluminium from all food sources at 1 mg/kg body weight per week [58].

Although most of the aluminium is eliminated through the kidney, this is a slow process and due to the long half-life a net-accumulation occurs. The lifelong body burden of aluminium is about 1% to 2% of the resorbed dose, which is estimated as 5 to 60 mg of aluminium [60,61], and with a higher risk in certain occupations [59]. Most of the aluminium is stored in the skeletal system, and about 1% is stored in the brain [62,63]. During a regular 3-year AIT cycle consisting of 8 applications per year, and with an allergen containing 0.5 mg aluminium per dose, an estimated total dose of 12 mg aluminium is administered. Calculating conservatively 2% retention, this would result in a life-long accumulating dose of 0.24 mg aluminium from AIT. Suppl. Table 2 illustrates differences in AIT regimens recommended by different providers.

The following aspects need to be considered:

- The most known **local reactions** are the development of granuloma. This is dependent on the type of alum and extracts, and the application [64,65].
- **Sensitization**. Contact allergies to aluminium are rare [66-68], but delayed type hypersensitivity may play a role in granuloma formation [64].
- There is a debate that Al(OH)3 may increase the allergy risk to the adsorbed allergen [69]. This is primarily based on animal models where Al(OH)3 is used as a Th2 adjuvant. However, it is difficult to extrapolate from the mouse to the human situation. Vaccinated patients uncommonly develop an IgE-mediated allergic response to the vaccine-antigen [70,71], except perhaps food allergens [72]. No evidence was reported that typical childhood vaccines such as M. bovis Bacille Calmette-Guerin, pertussis, influenza, measles, mumps, rubella or smallpox pose a risk for the later development of atopy [73] which, however, are mostly not adjuvanted with aluminium [71]. Furthermore, a long term effect of Al(OH)3 containing AIT, like with other adjuvants, is the induction of an IgG response (mostly IgG1, IgG4, much less IgG2 and 3) with a relative reduction of respective IgE antibodies [2,74,75].

- **Acute toxicity**. As a consequence of high aluminium exposure symptoms of acute toxicity including neurotoxic effects (encephalopathy), bone marrow effects (anaemia), and on reproduction have been extensively studied in animal models. In humans, acute toxicity was particularly observed in patients with chronic kidney disease following long-lasting haemodialysis: This syndrome is known as *dialysis encephalopathy syndrome* (DES) and occurred particularly in the 1970´s due to exorbitant aluminium uptake from the use of aluminium in the dialysis bath. Patients reached plasma levels between 200 to 500 μg/l associated with onset of brain malfunction at >30 μg/l [62,65]. There have been no phamacovigilance signals for acute toxicity linked to AIT.
- **Long-term toxicity**. There is a debate about the development of breast cancer, Alzheimer's disease, multiple sclerosis, autoimmunity [76,77] and other diseases in the context of aluminium burden. The German Institute of Risk Assessment (BfR) could not detect a relationship between the increase of aluminium intake from foods, medication, or cosmetic agents and the development of Alzheimer's disease [78], while a recent meta-analysis determined a 71% increased risk (OR: 1.71, 95% confidence interval (CI), 1.35–2.18) [79]. The use of newer staining methods like Lumogallion [80] have demonstrated to be useful to trace aluminium in tissues and may contribute to the necessary collection of more evidence.
- As much more data and studies are available for Al(OH)3 than for any other adjuvant in AIT (which need to be studied in more detail), the detailed description of current knowledge on Al(OH)3 may give the impression of an unfavourable benefit risk balance as an adjuvant, however no definite conclusions can be drawn at this point in time. Recent data derived from a model for aluminium toxicokinetics in rats give hope that individual vaccinations also in human tissue may not lead to measurable changes in the aluminium load.
- After several years of subcutaneous immunotherapy a substantial, but clinically not relevant increase in the aluminium concentration in the bone has to be expected. Reliable extrapolations from results in rats to humans will be possible with the help of a physiology-based model under development. However, the aluminium toxicity data combined with manifold repeated injections of Al(OH)3 in AIT (see suppl. Table 2), prompt us to carefully monitor the known and emerging pros and cons of all adjuvants. Many vaccines (e.g. diphtheria, tetanus, pertussis, hepatitis B, pneumococcal and meningococcal vaccines) contain Al(OH)3, because an effective vaccination would not be possible without this adjuvantation. The authors do not question the usefulness of these vaccines in principle,

are convinced of the survival benefit for mankind and reject any ongoing anti-vaccine discussion.

5. Monophosphoryl Lipid A

MPLA, precisely 3-O-desacyl-4'-monophosphoryl lipid A (MPL®), is a low-toxicity derivative of the lipid A region of lipopolysaccharide (LPS), that retains the immunologically active lipid A portion of the parent molecule. While the toxicity associated with LPS prohibits its clinical use, MPL has been developed as a vaccine adjuvant in anti-infectious, anti-cancer vaccines [81] (for instance, MPL is contained in the FDA- and EMA-approved marketed Human Papilloma Vaccine by GSK) and in AIT, allowing lower injection numbers mimicking rather the vaccine approach.

MPL is extracted from lipopolysaccharide (LPS or endotoxin) produced by the Re mutant of a rough strain Salmonella minnesota R595. Lipid A, a disaccharide with fatty acid side chains, is the component responsible for the endotoxic activity of LPS. Removal of one phosphate group from lipid A produces MPL (*alias MPLA*) which has reduced toxicity while retaining the ability to stimulate the immune system via TLR4. Synthetic lipid A (MPLAs) from *E. coli* is produced synthetically.

MPL in mouse studies [82] skews the immune response toward Th1 and Treg pathways, and it has been suggested that MPL improves vaccine immunogenicity by enhancing APC maturation. MPL, like CpG-ODN, imidazoquinolines and adenine derivatives acting via innate sensors represent improvements in AIT by interfering with pathogenic Th2 cells and promoting Th1 differentiation [83].

Both LPS and MPL are TLR4 agonists. TLR signalling is involved in activating innate and adaptive immune responses and plays a critical role in inflammation-induced diseases. Dysregulation of this signalling pathway can result in disturbance of epithelial layer homeostasis, caused by chronic inflammation and excessive repair responses. MPL and several other agents have been approved for anti-cancer vaccines as there is now substantial evidence for the benefit of targeting of this pathway in cancer [84].

LPS and MPLA signal through TLR4 which has two different TLR adaptors, MyD88 and TRIF. The reduced toxicity of MPLA is attributed to the preferential recruitment of TRIF upon TLR4 activation, resulting in decreased induction of inflammatory cytokines.

MPLAs activates TLR4 but does not activate TLR2 reflecting the high purity of this synthesized compound. MPLAs contains 6 fatty acyl groups, while MPL purified from bacteria contains a mixture of 5, 6, and 7 acyl lipid A.

Combining distinct immune stimulants in adjuvants can even further improve the quality of the immune response to the vaccine. A unique mechanism of molecular and cellular synergy between MPL, and a saponin, QS-21, the constituents of the Adjuvant System AS01, has been reported [85]. AS01 is part of the first malaria vaccine candidate and a herpes zoster vaccine that has recently received marketing authorization in a centralised procedure throughout the EU (21.3.2018). This mechanism, previously described for infections, illustrates how adjuvants trigger naturally occurring pathways and may improve the efficacy of AIT. Vice versa, the adsorption of allergoids and MPL to MCT in formulations for use in AIT suggested that it could be an alternative adjuvant depot for some infectious disease antigens [86]

Likewise, attempts were made to combine MPL with aluminium salts in the adjuvant system AS04 in papilloma vaccines [87]. More recently, it was demonstrated that combining MPL plus aluminium salts, or MPL plus muramyl-dipeptide (MDP), a NOD-like receptor (NLR) agonist exerted additive effects on the magnitude and quality of humoral responses towards HIV envelope antigens [88].

Due to the dual action of stimulating the immune system, a tyrosine-absorbed and MPL adjuvanted AIT was clinically effective after only four injections given pre-seasonally [89] and, in another study, contributed to the control of asthma during the pollen season [90]. An ultra-short course of ragweed MATA MPL (short ragweed pollen allergoid adsorbed to L-Tyrosine + MPL) was efficacious in reducing allergy symptoms in patients with seasonal allergic rhinitis and was well tolerated [91]. Ultra-short grass pollen AIT adjuvanted with MPL achieved specific bronchial tolerance as well as increased IgG4 levels (median before SCIT 0.34 to 11.4 kU/L after SCIT), whereas the total and specific IgE levels remained unchanged [92]. Especially in the presence of MPL, the allergenicity of an employed allergoid was sharply reduced when compared to the native allergen, while its immunogenicity was largely retained [93]. Booster AIT, using MCT-absorbed allergoids containing the adjuvant MPL, effectively prevented re-occurrence of symptoms in patients with grass pollen-induced allergic rhinoconjunctivitis who had completed a successful course of any grass pollen AIT at least 5 years before enrolment, compared to control patients who received symptomatic medication [94].

The following aspects need to be considered:

- The most common adverse effects of MPL adjuvanted AIT are transient and local, such as redness, swelling and pruritus at the injection site [95].
- However, also anaphylactic shock after administration of a pollen extract allergoid adsorbed onto L-tyrosine adjuvanted with MPL-4 has been described [96].

- Since MPL was introduced only in 1999, more data are needed to reveal any potential toxicity.

Overall, detoxified lipopolysaccaride (MPL-A), MPLAs, CpG-ODNs, imidazoquinolines and adenine derivatives acting via innate sensors represent improvements in therapeutic vaccinations for allergy as they are able to interfere with pathogenic Th2 cells with eventual induction of Th1 differentiation and enhancing IgG responses [83,97]. Furthermore, the use of explicit anti-Th2 adjuvants like MPL [89,98] instead of adjuvants like aluminium compounds [69] might well help to improve current AIT protocols, potentially also of SLIT [99].

6. Microcrystalline tyrosine

Tyrosine is an amino acid that in crystalline form can be used as a biodegradable adjuvant with depot effect. In a mouse model, MCT was recently compared head-to-head with Al(OH)3 [33], where it induced fewer anaphylactic reactions. In the same paper the immune mechanism of MCT as an adjuvant was addressed for the first time. In analogy to Al(OH)3, MCT provoked caspase-dependent secretion of IL-1β from cultured human monocytes, and in a model with immune-signalling-deficient and TCR-transgenic mice it was concluded that the inflammasome activation did not affect functionally the innate inflammatory or specific immune responses. In contrast to the LPS-derived MPL, MCT does not act via TLR4 signalling [33].

MCT induced in mice less IL-4 and IgE formation than aluminium. It is also applied safely in preclinical models of malaria vaccines [100,101]. Furthermore, MCT has been shown to be beneficial in influenza vaccination when compared to Al(OH)3 [102], where it enhanced antibody responses towards this vaccine.

When AIT effects on IgG4 induction were compared among the non-adjuvanted US product (Hollister-Stier®, Spokane, WA, US), and adjuvanted European products either using MCT (Tyrosine®, AllergyTherapeutics, UK) or Al(OH)3 (Novo-Hellisen®, AllergoPharma, Reinbeck, Germany) (Park), the US product showed the highest potency in inducing IgG4 [103]. However, in this study only patients without adverse side effects were included, thereby precluding any conclusions about simultaneous safety [103].

The following aspects need to be considered:

- At present, and since its introduction into AIT in 1970, there are no specific safety concerns known for MCT.
- It can be anticipated that this fully biodegradable adjuvans will also in future studies not reveal side effects.

7. Calcium-Phosphate

As an adjuvant calcium phosphate was developed 40 years ago as an adjuvant [104]. It has been included in vaccines against various infectious diseases such as diphtheria, tetanus, pertussis and poliomyelitis. It was shown to be well tolerated in humans, and even more efficacious than Al(OH)3 when used as part of a booster vaccine for DT (diphtheria/tetanus) [105].

Approved by the World Health Organization, CaP was further used in combination with allergens for hyposensitization purposes, based on the observation that it induces IgG, but not IgE responses. CaP is currently commercially available in Europe as a component of subcutaneous allergy vaccines in combination with grass pollen or mite extracts [106], [107] The aqueous allergen extracts are adsorbed onto the particulate CaP microcrystals. Allergen loading is thus thought to occur by passive adsorption, but also following encapsulation during particle formation. As a well-tolerated adjuvant, CaP has been proposed as a substitute to aluminium-based adjuvants in allergic humans and dogs [108]. A review of the present evidence suggests that CaP particles re-introduce a more balanced immune response when compared with aluminium salts, known to elicit a Th2 biased humoral immune response [105], [82].

Mechanisms of CaP in AIT include a depot effect with a slow release of the allergen. In addition, the adsorption of allergens onto CaP microcrystals as particles also facilitates the uptake by phagocytic cells (i.e. monocytes, macrophages, DCs), thereby enhancing the immunogenicity of protein allergens, with the induction of strong IgG responses [109]. As a mineral adjuvant, CaP also induces the NALP3 inflammasome, resulting in the secretion of IL1β and IL-18 pro inflammatory mediators.

The following aspects need to be considered:

- CaP is a compound present in many living organisms. As such, it is biocompatible and well tolerated by most patients. Common side effects include local reactions at the site of administration.
- More data are needed to exclude any potential toxicity.
- More studies are needed to support its efficacy as compared to other adjuvants.

Conclusion

AIT is applied in patients who are hypersensitive to an allergen and at risk for adverse immediate type reactions. However, quality, efficacy, safety and tolerability of AIT as the only disease-modifying treatment option are key. All reviewed types of marketed adjuvants precipitate the

allergen as a depot at the injection site thereby reducing the risk for systemic anaphylaxis, and also prolonging their availability for the immune cells.

The Task Force's review further revealed that, at present, only a limited number of adjuvants are applied in AIT vaccines. In marketed formulations aluminium compounds are predominant in Europe albeit the fact that aspects concerning health safety of aluminium have been controversially discussed. The immune mechanisms of the major adjuvants, Al(OH)3, MPL and MCT have only been addressed in recent years (while others are still missing), and explain their reported contrasting immune profile: aluminium, MCT and MPL induce high levels of blocking antibodies and regulatory T-cells; aluminium hydroxide initially boosts a Th2 response, while MPL and MCT induce early skewing towards a Th1 response; all adjuvants induce varying inflammation suggesting a hierarchy of biocompatibility MCT>CaP>MPL>Al(OH)3.

The preclinical pipeline is filled with interesting novel options in terms of adjuvants and carrier systems, and immune-modifying molecules, being more biocompatible and allowing development of improved immunization schedules with greater comfort for the patient. All reported strategies are of the highest importance to improve the insufficient adherence of patients in AIT independent of route of administration as SCIT or SLIT, resulting in only 18% of users reaching the minimal 3 year course duration in an earlier study [110], while in studies with other products adherence rates up to 50% were reported [111],[112]. The discussion is ongoing whether shorter treatment regimens could improve adherence [113], as most dropouts (with alumiunium or tyrosin adjuvanted products) occurred already in the first year [111], paradoxically with cluster build-up and rush schemes, younger age, and, interestingly, to longer disease duration [114]. In SLIT, forgetfulness may be the most important reason for dropouts [115]. Overall, adherence is a severe problem in all of the currently marketed AIT products, underlining the need for optimising AIT with novel adjuvants and enhanced efficacy towards true vaccine concepts.

Legends

Figure 1: Adjuvants enhance B- and T- cell responses to the antigen by various mechanisms. Adjuvanted antigens exploit activation of Toll like receptors (TLRs) on B cells, crosslinking of B cell receptors (BCR) by particles such as VLPs, and depot formulation to prolonge antigen stimulation. Further, adjuvanted antigen depots prolonge stimulation of DCs, targeting and enhancing uptake by dendritic cells (DCs), finally leading to activation of T-cells, which then help B-cells to become plasma cells. MPLA_ monophosphoryl lipid A ; CaP, calcium phosphate ; MCT, microcrystalline tyrosine.

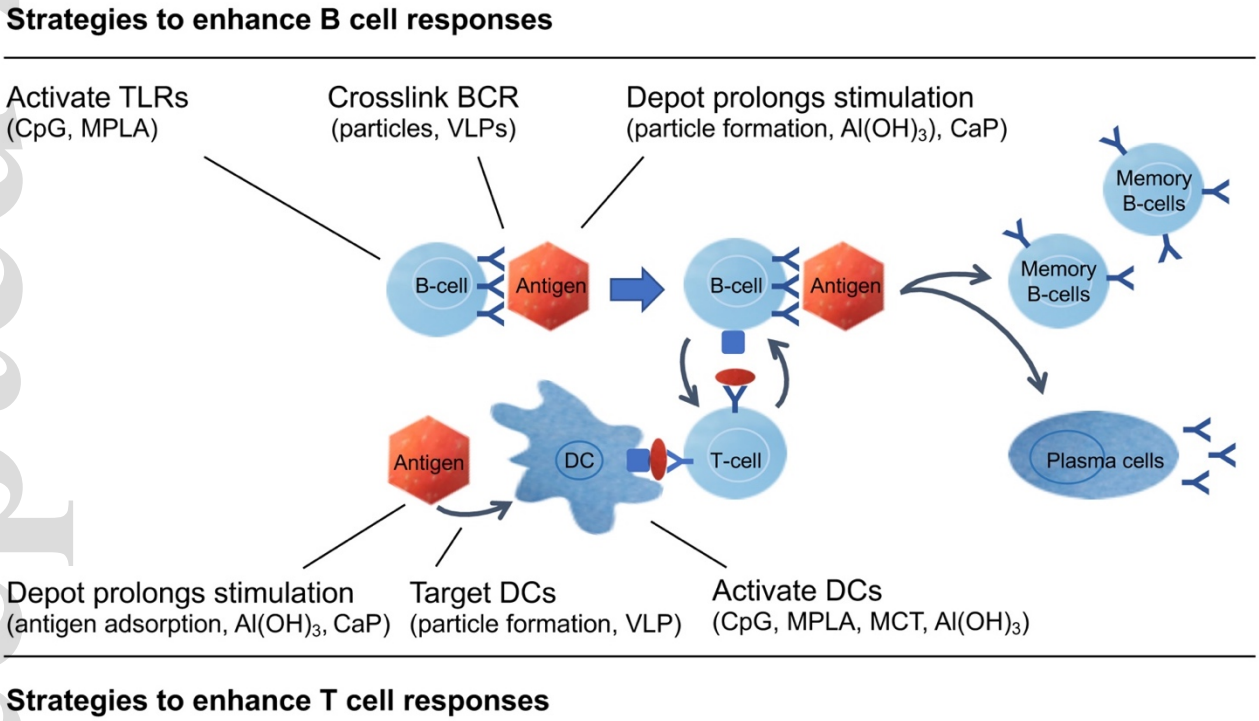

Figure 2. Adjuvants in grass pollen AIT marketed in Europe. A) Percentage of products using Al(OH)3, MCT, CaP, or MPLA as adjuvant in grass AIT; B) Average number of injections with grass pollen AIT products recommended by the industry for administration schemes over 3-5 years (data from suppl. Table 2).

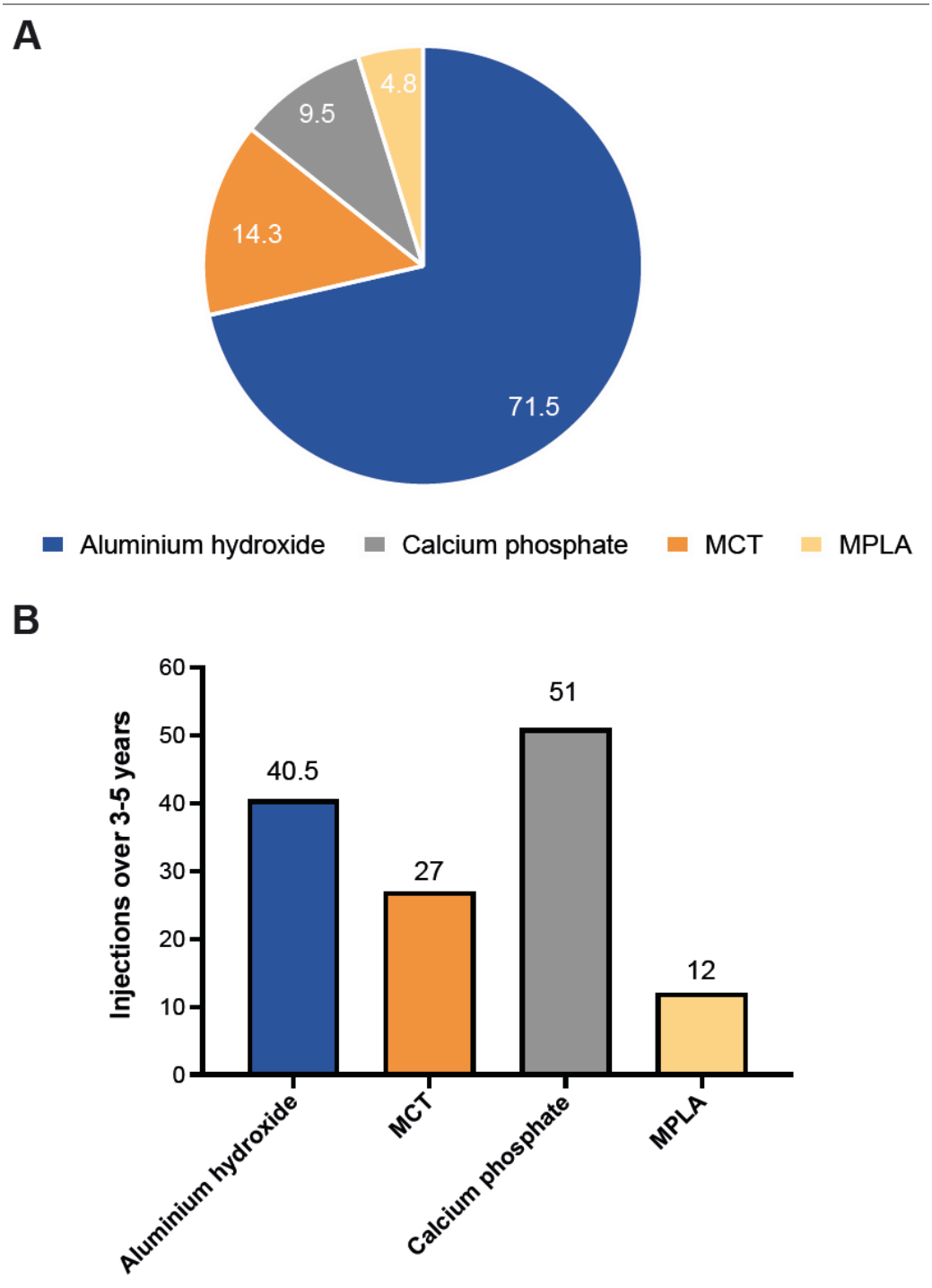

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Table 1: The contents and specification of AIT products

Components	*Characteristics*	*Chemical modification*
Allergen extracts	*Remain aqueous, native; used in SLIT and in United States for SCIT*	• *none*
Allergoids	*Chemically modified allergens with reduced IgE binding, and enhanced immunogenicity, used for SCIT in Europe*	• *Formaldehyde* • *Glutaraldehyde* • *Calcium cyanate*
Adjuvants *)	*Achieve physical allergen depot for enhanced safety and immunogenicity, used for SCIT in Europe*	• *Aluminium hydroxide* • *Calcium phosphate* • *Microcrystalline Tyrosine (MCT)* • *Monophosphoryl Lipid A (MPLA)*

**) Labeling obligatory in EU*

Table 2: Detailled list on product informations for subcutaneous AIT products by industry.

Industry	Brand name of product	Market area	Chemistry of the adjuvant				Physical properties of the adjuvant		
			Chemical name	Synonyms/ abbreviations	linear chemical formula	molecular weight	form	density	particle size
Allergopharma	Allergovit Grasses	Europe	Aluminum-hydroxide	Alum	$Al(OH)_3$	78.0 g/mol	in suspension	n.discl.	n.discl.
ALK	Alutard SQ	n.discl.	Aluminium hydroxide, Sodium chloride Sodium hydrogen bicarbonate Phenol, water for injections	n.discl.	$Al(OH)_3$	n.discl.	n.discl.	n.discl.	n.discl.
ALK	ALK7/Start SQ		Aluminium hydroxide, Sodium chloride Sodium hydrogen bicarbonate, Phenol	n.discl.	$Al(OH)_3$	n.discl.	n.discl.	n.discl.	n.discl.
ALK	AVANZ®	n.discl.	Aluminium hydroxide Sodium chloride Sodium hydrogen carbonate Phenol Water for injections	n.discl.	$Al(OH)_3$	n.discl.	n.discl.	n.discl.	n.discl.
ALK	Pangramin Ultra	n.discl.	Aluminium hydroxide Sodium chloride Sodium hydroxide/ Hydrochloric acid Phenol Water for injections	n.discl.	$Al(OH)_3$	n.discl.	n.discl.	n.discl.	n.discl.
ALK	Aquagen SQ	n.discl.	Mannitol Water for injections	n.discl.	n.discl.	n.discl.	n.discl.	n.discl.	n.discl.

ALK	IRIS	n.discl.	Phenol Mannitol Aluminium hydroxide Sodium hydrogen carbonate Sodium chloride Water for injections	n.discl.	n.discl.	n.discl.	n.discl.	n.discl.	n.discl.
Allergy Therapeutics	Pollinex Grass, TA Graser top, MATA PFS	globally available	Microcrystalline Tyrosine	MCT, L-tyrosine	C9H11NO3	181.19 Da	Crystalline	n.discl.	20µm
Allergy Therapeutics	Pollinex Quattro Grass	globally available	Monophosphoryl Lipid A	MPL, MPL-A	C94H176N2O22P1 (A)	1715‡ Da	Micelle Formulation	n.discl.	< 144nm
Allergy Therapeutics	Pollinex Quattro Grass	globally available	Microcrystalline Tyrosine	MCT, L-tyrosine	C9H11NO3	181.19 Da	Crystalline	n.discl.	20µm
Allergy Therapeutics	Tyrosine TU top Grass	globally available	Microcrystalline Tyrosine	MCT, L-tyrosine	C9H11NO3	181.19 Da	Crystalline	n.discl.	20µm
HAL Allergy BV	Purethal Grasses	Europe	Aluminium hydroxide	Aluminic acid; Aluminic hydroxide; Aluminium(III) hydroxide; Aluminium hydroxide; Aluminum trihydroxide; Hydrated alumina; Orthoaluminic acid	Al(OH)3	78.0 g/mol	white amorphous powder	2.42 g/cm^3	n.discl.
LETI	Depigoid grass	n.discl.	Aluminium hydroxid	Alhydrogel	$Al(OH)_3$	78.0 g/mol	depigmented, glutaraldehyde polymerized, chemically modified allergenic extract of Phleum pratense;Hydrogel (white gelatinous precipitate)	n.discl.	n.discl.
	Depiquick		Aluminium hydroxid						
LOFARMA SpA	LAIS-in	Europe	Calcium phosphate	n.discl.	n.discl.	n.discl.	depigmented, glutaraldehyd	n.k.	about 100 nm

ROXALL	CLUSTOID	Germany-Austria-Italy	Aluminium hydroxide	Alhydrogel 2%	$Al(OH)_3$	78.0 g/mol	Hydrogel (white gelatinous precipitate), Fibrous primary particles	2.42 g/cu cm	1-10 µm
ROXALL	Deposit	Germany	Aluminium hydroxide	Alhydrogel 2%	$Al(OH)_3$	78.0 g/mol	Hydrogel (white gelatinous precipitate), Fibrous primary particles	2.42 g/cu cm	1-10 µm
ROXALL	Allergovac depot	Spain-Portugal-Italy	Aluminium hydroxide	Alhydrogel 1.3%	$Al(OH)_3$	78.0 g/mol	Hydrogel (white gelatinous precipitate), Fibrous primary particles	2.42 g/cu cm	1-10 µm
ROXALL	Allergovac Polimerizado	Spain-Portugal-Italy	Aluminium hydroxide	Alhydrogel 1.3%	$Al(OH)_3$	78.0 g/mol	Hydrogel (white gelatinous precipitate), Fibrous primary particles	2.42 g/cu cm	1-10 µm
STALLERGENES	Alustal®	n.discl.	Aluminium hydroxide	n.discl.	$Al(OH)_3$	78 g/mol	n.discl.	n.discl.	n.discl.
STALLERGENES	Phostal®	n.discl.	Calcium phosphate	n.discl.	$Ca_3(PO_4)_2$	310 g/mol	n.discl.	n.discl.	n.discl.

*) unique numerical identifier assigned by the Chemical Abstracts Service)

**) permanent identifier for a depositor-supplied molecule found in the PubChem Substance database

***) Molecular Design Limited Number.

1.) n.discl. - non d+J4isclosed

2.) n.k. not known

3.) mgeq: mg equivalent. The group 5 major allergen content is estimated before allergoidization. The adjuvant is aluminiumhydroxide, the Al3+ content has been calculated.

4.) DPP = biological unit (1 DPP corresponds to 1 HEP of native allergen extract after depigmentation and polymerization /allergoidisation; HEP, histamine equivalent in prick testing

Table 3. The clinical pipeline of adjuvants in allergen specific immunotherapy: registered clinical trials. A systematic review of the literature was performed in PubMed in 06/2018 for the preclinical trials using the terms “delivery system”, “adjuvant” and “allergen”, and by search of databases such as European clinical trials database EudraCT and ClinicalTrials.gov, using the term “allergy” and restricting to intervention trials.

Type of Adjuvant	Classification	Allergen formulation	Route	No. of studies	phase
Mineral Salts	Aluminium salts	Allergen extracts	SCIT[1)]	> 40	2/3/4
		Allergoids [2)]	SCIT	2	2
		Allergen fusion Proteins	SCIT	4	2
		Hypoallergens	SCIT	2	2/3
		VLPs [3)] displaying allergen molecules	SCIT	1	2b
	Calcium phosphate	Allergen extracts	SCIT	2	4
Amino acids	Tyrosine	Allergen extracts, and Allergoids	SCIT	>15	1/2/3/4
TLR activators	TLR4 agonist MPL [4)]	Allergen extracts, Allergoids	SCIT	>15	1/2/3
	CpG ODN [5)]	Allergen extracts	SCIT	1, withdrawn	2
	TLR9 agonist QbG10	Allergen extracts	SCIT	4	1/2
Adjuvanted mucosal applications					
TLR activator	MPL	Allergen extract	SLIT	1	1
Conjugate	Mannan	Allergoids	SLIT [6)]	2	2
Microparticles	Cellulose	Allergen extracts	intranasal, SLIT	1	2/3
	Chitin	Allergen extracts	intranasal	1	1/2
Adjuvanted applications via the skin					
Microparticles	PLA [7)]	Allergen extracts	epicutaneous	1	1
Microparticles	Silver	Allergen extracts	epicutaneous	1	2

Footnotes:

1.) SCIT, subcutaneous allergen-specific immunotherapy

2.) Allergoids: Allergen extracts being polymerized by glutaraldehyde treatment, carbamylated or conjugated to mannan.
3.) VLPs, virus-like particles
4.) MPL, Monophosphoryl lipid A
5.) CpG ODN, cytosine-phosphate-guanine oligodeoxynucleotides
6.) SLIT, sublingual allergen-specific immunotherapy
7.) PLA, polylactic acid